3' untranslated regions, introns, poly A addition site and transcription terminators. Modification within the expression cassette does not prevent expression of the gene. In one preferred embodiment, this can be accomplished by first chemically modifying a piece of DNA which contains an expressible sequence such

5   as an entire plasmid. Thus the attachment can be to sequences either within or outside the sequences required for expression.

Gene Transfer Enhancing Signals

In a preferred embodiment, a chemical reaction can be used to attach a gene

10   transfer enhancing signal to a nucleic acid. The gene transfer enhancing signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies the nucleic acid complex for more efficient delivery to a location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the cellular or tissue location of the foreign gene, the expression of the

15   foreign gene can be enhanced.

The gene transfer enhancing signal can be a protein, peptide, lipid, steroid, sugar, carbohydrate, (non-expresssing) polynucleic acid or synthetic compound. The gene transfer enhancing signals enhance cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other

20   intracellular vesicles.

Nuclear localizing signals enhance the targeting of the gene into proximity of the nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T antigen NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors

25   such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta. The nuclear transport proteins themselves could also function as NLS's since they are targeted to the nuclear pore and nucleus. For example, karyopherin beta itself could target the DNA to the nuclear pore complex. Several peptides have been derived from the SV40 T antigen. These include a short NLS SEQ ID NO: 1 (H-

30   CGYGPKKKRKVGG-OH) or long NLS's SEQ ID NO: 2 (H-CKKKSSSDDEATADSQHSTPPKKKRKVEDPKDFPSELLS-OH and SEQ ID NO: 3 H-CKKKWDDEATADSQHSTPPKKKRKVEDPKDFPSELLS-OH). Other NLS peptides have been derived from M9 protein SEQ ID NO: 4

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(CYNDFGNYNNQSSNFGPMKQGNFGGRSSGPY), E1A SEQ ID NO: 5 (H-CKRGPKRPRP-OH), nucleoplasmin SEQ ID NO: 6 (H-CKKAVKRPAATKKAGQAKKKKL-OH),and c-myc SEQ ID NO: 7 (H-CKKKGPAAKRVKLD-OH).

5   Signals that enhance release from intracellular compartments (releasing signals) can cause DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle

10   such as the nucleus. Releasing signals include chemicals such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

          Cellular receptor signals are any signal that enhances the association of the

15   gene with a cell. This can be accomplished by either increasing the binding of the gene to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asiologlycoproteins or galactose residues. Other proteins such as insulin, EGF, or

20   transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with sulfhydryl or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids fatty acids, cholesterol, dansyl compounds,

25   and amphotericin derivatives. In addition viral proteins could be used to bind cells.

          In a preferred embodiment, a complex is formed by using a modifying chemical attachment for attaching a plurality of compound to a nucleic acid in an amount sufficient to change the tertiary structure of the nucleic acid. The change in tertiary structure allows for more efficient delivery of the complex to a cell, *in vivo*,

30   when compared to compound attachment where the attachment is less strong such as ionic bonding.

          In a preferred embodiment, a process for nucleic acid delivery includes preparing a nucleic acid molecule having an expressible sequence. A compound is

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attached to the nucleic acid molecule within the expressible sequence which allows more than 40% expression of the expressible sequence as compared to the nucleic acid without compound attachment. The nucleic acid is delivered to a cell where the expressible sequence may be expressed.

5   In a preferred embodiment a signal is covalently attached to a plasmid (i.e. circular polynucleotide) randomly at a position either within or outside of the expressible sequence of the plasmid.

In another preferred embodiment the signal is attached to a polynucleotide either within or outside the expressible sequence and the signal is selected from a

10   group of compounds that stimulate an enhanced immune reaction against the protein encoded by the expressible sequence.

In another preferred embodiment a polycation is covalently attached to a polynucleotide resulting in DNA compaction and negatively charged particle formation. The attachment of the polycation allows for the formation of DNA

15   particles that have a different tertiary (3-dimensional) conformation than particles formed by non-covalent interactions. In one This indicates that charge neutralization is not required and particle formation is not the result of charge mediated DNA condensation.

20   **Example 1.**

DNA covalently modified (alkylated) with rhodamine is efficiently expressed in COS 7 cells following transfection.

25   Methods.

DNA labeling - Rhodamine molecules were covalently attached to plasmid DNA (pCILuc) encoding the reporter gene luciferase through an alkylation reaction. The plasmid, pCILuc was mixed with *Label*IT® Rhodamine (Mirus Corporation, Madison WI) at three different ratios (1:0.2; 1:0.1; 1:0.05) (wt:wt) and incubated for 30

30   minutes at 37°C. Rhodamine-labeled DNA was purified and concentrated by ethanol precipitation. Rhodamine-labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all rhodamine-labeled DNA was observed (data not shown).

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Luciferase expression - For gene expression studies rhodamine-labeled DNA was complexed with the transfection reagent TransIT LT-1 (Mirus Corporation) at a 1:3 (wt : vol) ratio and added to COS 7 cells according to manufacturer's recommendations. Transfected cells were grown at 37°C for 48 hours and harvested

5 into luciferase buffer (0.1M KPO4, pH 7.8; 1mM DTT; 0.1% Triton X-100). Cell lysates were assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

Results.

10 *LabelIT*® rhodamine facilitates the attachment of rhodamines primarily at guanine nucleotides so labeling occurs throughout the plasmid. The different levels of labeling that was used results in the covalent attachment of about 1 rhodamine per 30 – 120 base pairs of DNA. Thus the protein coding sequence of the luciferase gene cassette would be expected to contain a large number of covalently modified guanine residues

15 (~12 – 50 labels). To determine the effect of the covalent attachment of rhodamines on gene expression, luciferase activity of the modified plasmid DNA was compared to the similarly transfected unmodified plasmid DNA. From these experiments we found that covalently modified pCILuc was expressed at levels similar to unmodified pCILuc.

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DNA Transfected	Luciferase Expression in liver (Relative to unmodified pCILuc)
Naked DNA (pCILuc)	1.0
pCILuc-Rhodamine (0.2 : 1 labeling ratio, *LabelIT*®rhodamine: pDNA)	0.47
pCILuc-Rhodamine (0.1 : 1 labeling ratio, *LabelIT*®rhodamine: pDNA)	0.80
pCILuc-Rhodamine (0.05 : 1 labeling ratio, *LabelIT*®rhodamine: pDNA)	0.88

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Example 2.

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DNA chemically modified with DNP using a modifying chemical non-covalent attachment (cis-platinum) is expressed in COS7 cells following transfection as efficiently as unmodified DNA.

10   Methods.

DNA labeling – dinitophenol (DNP) molecules were attached to plasmid DNA (pCILuc) encoding the reporter gene luciferase through a cis-platinum reaction. The plasmid, pCILuc was mixed with the labeling reagent (Versitag, NEN) at a 0.1:1 ratios (vol:wt) and incubated for 30 minutes at 85°C. DNP-labeled DNA was purified

15   and concentrated by ethanol precipitation.

Luciferase expression - For gene expression studies DNP-labeled DNA was complexed with the transfection reagent TransIT LT-1 (Mirus Corporation) at a 1:3 (wt : vol) ratio and added to COS7 cells according to manufacturer's recommendations. Transfected cells were grown at 37°C for 48 hours and harvested

20   into luciferase buffer (0.1M KPO4, pH 7.8; 1mM DTT; 0.1% Triton X-100). Cell lysates were assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

Results.

25   Versitag-DNP facilitates the attachment of DNP molecules primarily at guanine nucleotides so labeling occurs throughout the plasmid. To determine the effect of the non-covalent DNP attachment on gene expression, luciferase activity of the modified plasmid DNA was compared to the similarly transfected mock-labeled plasmid DNA. From these experiments we found that DNP modified pCILuc using a modifying

30   chemical non-covalent attachment was expressed at about 50% that of unmodified pCILuc.

<u>DNA Transfected</u>   <u>Luciferase Expression COS7 cells.</u>

21

	(Relative to mock pCILuc)
Mock labeled DNA (pCILuc)	1.0
pCILuc-DNP	0.49

(0.1 : 1 labeling ratio, Versitag-DNP: pDNA)

Example 3

DNA covalently modified (alkylated) with digoxin is efficiently expressed in mouse liver hepatocytes following in vivo delivery.

Methods:

*DNA Labeling - Labe/*IT®digoxin (Mirus Corporation, Madison WI) was used to covalently attach digoxin molecules to a plasmid DNA encoding the reporter gene luciferase (pCILuc). Three different labeling ratios were used to achieve varying amounts of plasmid labeling (0.1:1, 0.05:1, 0.025:1 wt:wt, labeling reagent to DNA). The digoxin labeled DNAs was purified and concentrated by ethanol precipitation. Labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all labeled DNA was observed (data not shown).

In vivo gene delivery – Digoxin-labeled and unlabeled pCILuc was delivered into mice via tail vein injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected (2.5 ml solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Animals were sacrificed 1 day after injections and the livers surgically removed. Livers were homogenized in luciferase buffer (0.1M KPO4, pH 7.8; 1mM DTT; 0.1% Triton X-100) and assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

Results:

The luciferase encoding plasmid DNA, pCILuc, was covalently labeled with varying amounts of digoxin and compared to unmodified pCILuc in *in vivo* gene delivery assays. In concordance with the in vitro transfections, covalently modified pCILuc expressed luciferase at least as efficiently as unmodified pCILuc.

DNA Injected	Luciferase Expression in liver (Relative to unmodified pCILuc)
Naked DNA (pCILuc)	1.0
pCILuc- digoxin (0.1 : 1 labeling ratio, *LabelIT*®digoxin : pDNA)	2.29
pCILuc- digoxin (0.05 : 1 labeling ratio, *LabelIT*® digoxin : pDNA)	4.67
pCILuc- digoxin (0.025 : 1 labeling ratio, *LabelIT*®digoxin : pDNA)	1.87

Example 4:

DNA covalently modified (alkylated) with biotin is efficiently expressed in mouse liver hepatocytes following in vivo delivery.

Methods:

DNA Labeling - LabelIT®biotin (Mirus Corporation, Madison WI) was used to covalently attach biotin molecules to a plasmid DNA encoding the reporter gene luciferase (pCILuc). Four different labeling ratios were used to achieve varying amounts of plasmid labeling. The biotin labeled DNAs was purified and concentrated by ethanol precipitation. Labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all labeled DNA was observed (data not shown).

In vivo gene delivery – Biotin-labeled and unlabeled pCILuc was delivered into mice via tail vein injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected (2.5 ml solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Animals were sacrificed 1 day after injections and the livers

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surgically removed. Livers were homogenized in luciferase buffer (0.1M KPO4, pH 7.8; 1mM DTT; 0.1% Triton X-100) and assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

5   Results:

The luciferase encoding plasmid DNA, pCILuc, was covalently labeled with varying amounts of biotin and compared to unmodified pCILuc in *in vivo* gene expression assays. Following intravascular delivery (tail vein injection) to liver hepatocytes, biotinylated pCILuc was expressed at least as efficiently as unmodified pCILuc.

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DNA Injected	Luciferase Expression in liver
	(Relative to unmodified pCILuc)
Naked DNA (pCILuc)	1.0
pCILuc-biotin (0.2 : 1 labeling ratio, *Labe*l IT® biotin : pDNA)	0.87
pCILuc-biotin (0.1 : 1 labeling ratio, *Labe*l IT® biotin: pDNA)	3.25
pCILuc- biotin (0.05 : 1 labeling ratio, *Labe*l IT® biotin: pDNA)	5.39
pCILuc- biotin (0.025 : 1 labeling ratio, *Labe*l IT® biotin: pDNA)	1.41

Example 5

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The covalent attachment of a peptide signal (nuclear localizing signal) to an expressible sequence enhances gene expression in vivo.

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Methods:

DNA Labeling.

A Nuclear Localizing Sequence peptide SEQ ID NO: 8 (NLS, CPKKKRKVEDG)
derived from the SV40 large T antigen and a control peptide were covalently attached
to a DNA reactive compound (*Label*IT®-Amine; Mirus Corporation). *Label*IT® is a
nitrogen mustard derivative that alkylates nucleic acid, thus forming a covalent bond
between the reagent and the nucleic acid. After attachment of peptides to *Label*IT®,
the *Label*IT®-NLS and the *Label*IT®-control peptide were reacted with plasmid DNA
(pCILuc) at the ratios indicated below. The DNA alkylation reaction facilitated by the
L *Label*IT® compounds results in covalent attachment of the peptides directly to the
DNA. In this alkylation reaction the peptides are covalently attached to sequences
throughout the plasmid both inside the expressible gene sequence and outside.

Intravascular Injections.

Ten micrograms of covalently modified or control DNA was injected into the tail vein
of ICR mice (Harlan Sprague Dawley) as previously described (Zhang et al. Human
Gene Therapy, 10:1735-1737). Twenty four hours after injection, livers were excised
and cell extracts were prepared and assayed for reporter gene activity (luciferase).

Results:

Plasmid DNA encoding the luciferase gene (pCILuc) was covalently modified via the
attachment of a peptide signal SEQ ID NO: 8 (nuclear localizing sequence;
CPKKKRKVEDG) or a control peptide SEQ ID NO: 9 (Mirus 017;
IAEYIPLETDLG) and injected into the tail vein of mice using an in vivo gene
delivery method. All covalently modified plasmid constructs were compared to
unmodified pCILuc for gene expression capabilities. Reporter gene expression
(luciferase) was assayed in the liver after 24 hours. Both sets of animals that received
DNA with covalently attached NLS peptides displayed levels of gene expression
higher than both naked DNA controls and DNA modified with a control (non-NLS
peptide). Two conclusions can be drawn from these results. 1) The covalent
attachment of peptides to expressible sequences does not inhibit gene expression as
compared to unmodified plasmid DNA; and 2) the attachment of a functionally active
peptide (i.e. NLS) augments gene expression.

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